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Effect of Nitrosamine (NNAT) on Embryogenesis: Evidence from a Study Using Avian Embryos Exposed to NNAT

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EFFECT OF NITROSAMINE (NNAT) ON EMBRYOGENESIS: EVIDENCE FROM A STUDY USING NNAT EXPOSED AVIAN EMBRYOS

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Background

- *N*-nitrosoatrazine (NNAT) forms in the acidic environment of the human stomach when nitrite (from nitrate) and atrazine are present together[1].
- NNAT is a nitrosamine, many of which are known carcinogens, but little is known about NNAT toxicity. The effects of NNAT on embryo development are virtually unknown [2].
- Chromosomal aberrations and an increase in mitotic index were observed in human lymphocytes exposed to NNAT at 1,000 to 10,000-fold lower concentrations than nitrate, nitrite, or atrazine alone[3] and NNAT has been shown to be mutagenic [4].
- We were among the first to account for the correlation of exposure to agrichemical mixtures from drinking water to birth defect rates in the 93 counties of Nebraska [5]

Hypothesis

We hypothesize that the embryos exposed to NNAT would have delayed development and increased mortality compared to unexposed embryos.

Method

Treatment, Incubation, and Analysis of Chicken Embryos

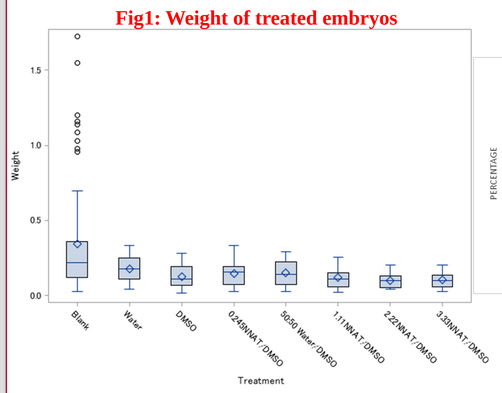
- For dosing, the eggshell was punctured on the broad, flat side of the egg. Eggs were administered 50 µL doses of NNAT in Dimethyl Sulfoxide (DMSO). Untreated eggs served as negative controls and eggs injected with DMSO served as vehicle controls. DMSO was controlled for by eggs injected with distilled water
- The different doses of NNAT administered were 0.245, 1.11, 2.22 and 3.33
- 330 eggs were incubated for 5 days in a forced air incubator at 38 ºC and 65-75% relative humidity

Data collection and analysis

- After 5 days of incubating the treated eggs the weight, morphology, and vital status of the embryos were obtained
- All data analysis was done using SAS 9.4
- It was observed that the position of the eggs in the incubator affected their weight from a linear regression analysis
- It was also observed from a Chi square analysis that the column arrangement of the eggs was associated to the vital status of the eggs.
- The effects of NNAT treatment on the weight of the embryos was obtained after controlling the effect of position in a centered ANCOVA
- The likelihood of death after treatment with NNAT was obtained after adjusting for column of the eggs in a multiple logistic regression
- The lethal dose of NNAT was determined using PROC PROBIT with a log10 transformation using SAS 9.4

Findings

Fig1: Weight of treated embryos



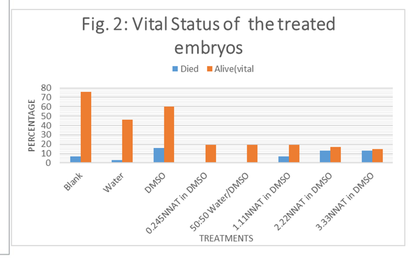


Table 1: Effect of treatment on the weight of embryo

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	13.45744989	1.49527221	37.21	<.0001
Error	241	9.68413811	0.04018315		
Uncorrected Total	250	23.14158800			

Table 2: Mean weight of the treated embryos

Parameter	Estimate	Standard Error	t Value	Pr > |t|
Blank	0.3430998831	0.02255601	15.21	<.0001
Water	0.1764897659	0.02955875	5.97	<.0001
50:50 Water/DMSO	0.1815501283	0.06339472	2.86	0.0046
DMSO	0.1261959807	0.02655123	4.75	<.0001
0.245NNAT in DMSO	0.1566642152	0.06339753	2.47	0.0142
1.11NNAT in DMSO	0.1280735693	0.04603997	2.78	0.0058
2.22NNAT in DMSO	0.1083519218	0.04603250	2.35	0.0194
3.33NNAT in DMSO	0.1001346495	0.06342173	1.58	0.1157
center	0.0045704343	0.00103479	4.42	<.0001

Table 3: Comparisons of the weight of the embryos among the treatment groups

Comparisons significant at the 0.05 level are indicated by ***.

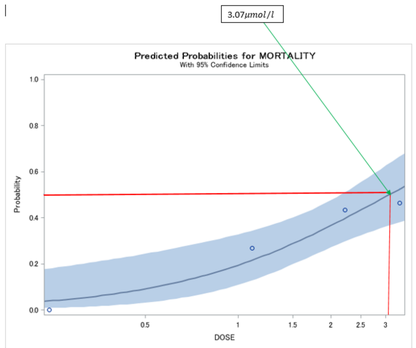
Treat_A_Incr Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
0.245NNAT/DMSO - 1.11NNAT/DMSO	0.03398	-0.20549	0.27345	
0.245NNAT/DMSO - 2.22NNAT/DMSO	0.05298	-0.18649	0.29245	
0.245NNAT/DMSO - 3.33NNAT/DMSO	0.06110	-0.21302	0.33522	
DMSO - Blank	-0.21529	-0.32181	-0.10876	***
DMSO - Water	-0.05208	-0.17357	0.06941	
1.11NNAT/DMSO - Blank	-0.22311	-0.37973	-0.06649	***
2.22NNAT/DMSO - Blank	-0.24211	-0.39873	-0.08549	***
3.33NNAT/DMSO - Blank	-0.25023	-0.45597	-0.04450	***

Table 4: Likelihood of embryo death after treatment with NNAT

Odds Ratio Estimates

Effect	Point Estimate	95% Wald Confidence Limits	
Blank vs DMSO	0.351	0.136	0.911
Water vs DMSO	0.249	0.068	0.909
1.11NNAT in DMSO vs DMSO	1.416	0.505	3.970
2.22NNAT in DMSO vs DMSO	2.905	1.167	7.228
3.33NNAT in DMSO vs DMSO	3.344	1.320	8.475
column	1.074	0.924	1.249

Fig. 3:Predicted probabilities for lethal dose of NNAT



Discussion

- It was found that the weight of the embryos differ significantly in at least 2 of the treatment groups (p<0.0001) (Table 1)
- NNAT decreased the weight of the embryos (0.245NNAT Wgt=0.16g, p=0.014, 1.11NNAT Wgt=0.13g, p=0.0058, 2.22NNAT Wgt=0.11g, p=0.019) (Fig1, Table 2)
- Embryos treated with DMSO had lower weight compared to the embryos treated with water and the untreated embryos (Fig1)
- The mean weight of embryos treated with NNAT differ significantly when compared to the untreated embryo (P<0.05) (Table 3)
- Higher mortality for embryos exposed to NNAT compared to unexposed embryos. (Fig 2)
- NNAT-exposed embryos were more likely to die compared to unexposed embryo (2.22NNAT vs. DMSO(OR: 2.91, 95% C.I. 1.17,7.23) and 3.33NNAT vs. DMSO (OR=3.34, 95% C.I. 1.32,8.47) (Table 4)
- The dose of NNAT required to kill 50% of the embryo is 3.07$\mu mol/l$ (95 C.I. 2.16, 7.14) (Fig 3)

Conclusion and Future Directions

- Developing chicken embryos were used in the present study to evaluate the teratogenic potential of *N*-nitrosoatrazine, a nitrosated form of the agrichemical atrazine
- NNAT has some teratogenic properties based on the weight and survival of chicken embryos
- A limitation to achieving these objectives is the low solubility of NNAT in water (290 mg/L) and the unclear toxic nature of DMSO to living tissues.
- A more suitable solvent for demonstrating the teratogenic properties of NNAT should be considered in future studies

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